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<b>13. ABSTRACT (Maximum 200 Words)</b> The purpose of this study is to identify genetic modifiers of cancer risk in women with BRCA1 and BRCA2 mutations. We are using two complementary strategies: 1) association studies in candidate genes from the immune surveillance and DNA damage response pathways and 2) a genome-wide scan using relative pairs with BRCA1 mutations to identify novel regions containing modifier genes. To date we have assembled a case-control sample set of 448 mutation carriers and a relative pairs set of 534 mutation carriers. We have completed a sequencing survey of a panel of immune surveillance genes and determined the population frequency of the variants we identified. We have examined a number of candidate genes and have data suggesting variants in TNF- $\alpha$ , IL-6, XPD and p53 may have a role in altering cancer risk in these high risk women. This work is important not only in leading to more refined cancer risk estimates for women with BRCA1 and BRCA2 mutations, but also will yield candidates for risk alleles in the general population and generate hypotheses for mechanisms that explain these effects. Once these mechanisms have been elucidated, these points in key pathways become excellent targets for preventative and therapeutic intervention.				
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## INTRODUCTION

The focus of this research study is the identification of genetic factors that influence cancer risk in women with BRCA1 and BRCA2 mutations. We have collected DNA and information from a large retrospective cohort of women with BRCA1 mutations. These were assembled into two, overlapping study samples: 1) a case control set where all study samples derive from women with BRCA1 mutations and cases those with breast cancer and controls are those carriers that have not developed breast cancer and 2) a relative pairs set where all samples are matched with at least one family member who also has a BRCA1 mutation. These sets are being used with two distinct methodologies to identify genetic modifiers of BRCA1 penetrance, including a candidate gene approach focused on a panel of genes involved in response to DNA damage and of genes important in modulating immune surveillance and a modified linkage approach to identify novel genes.

## PROGRESS REPORT

Task 1: Screening of all genetic variants in a series of candidate genes (Months 1-18).

a. Collection of DNA samples from all collaborators.

This task was completed primarily during year 1. The sample set from which the case-control set for analysis of candidate genes was constructed includes 656 women with germline *BRCA1/2* mutations. These samples were ascertained in a retrospective fashion AFTER identification of the through families with a history of breast and/or ovarian cancer at Creighton University, the Dana Farber Cancer Institute, The University of Michigan, Fox Chase Cancer Center, The University of Pennsylvania, The University of Utah, or Women's College Hospital (Toronto) between 1978 and 1997. The resulting case control sample of 448 women consists of 278 breast cancer cases and 170 matched controls.

The collection of relative pairs has been completed this year. We have ascertained data on 600 BRCA1 mutation carriers. Of those, 242 female mutation carriers from 51 families have a relative pair with a known mutation and a DNA sample that can be included in the analysis and these samples are in hand. Ninety individuals from 21 families were used for the chromosome 5q linkage analysis (see below and attached manuscript) and the remainder will be used in the upcoming year for validation of this finding and genotyping of candidate genes in the region.

Subtasks b-f (PCR amplification of variant fragments and microsatellites (b), separation with automated sequencer (c), checking of automated data (d), data analysis (e) and reanalysis if indicated (f)) are completed sequentially for each gene undergoing analysis before conclusions can be drawn so they are considered as a single task with the following analyses.

### Immune surveillance genes

We completed a comprehensive sequence analysis of 13 immune surveillance genes for the presence and frequency of 26 polymorphisms in a control population set in Year 1.

This year we have arrayed the case-control samples into 96-well microtiter plates and begun the analysis of a number of immune surveillance candidate genes (see below). We have optimized PCR conditions for all polymorphisms on the ABI 3100 capillary sequencer and have screened 13 genes/26 polymorphisms (14 novel) in the case control set and have found the following:

- 7 polymorphisms where the variant allele is present at >15% frequency in both groups  
IL-1 $\beta$ , IL1-RN (2), IL-2, IL-6, IL-10, CTLA4
- 6 polymorphisms where the variant allele is present at < 15% frequency in only one group  
TNF- $\alpha$  (2), TNF- $\alpha$ R (2), IL-12p35, CTLA4
- 11 polymorphisms where the variant allele is present at <15% frequency in both groups  
TNF- $\alpha$ (4), IL-1 $\alpha$ (2), IL-10, IL-12p35, IL-12p40(3), CTLA4

We are now completing the statistical analysis of these polymorphisms in for associations with case status and age of diagnosis. We have preliminary evidence from these analyses that polymorphisms in TNF- $\alpha$  and IL-6 may be associated with variable breast cancer risk in BRCA1/2 mutation carriers. These polymorphisms are now being analyzed in a validation set of an additional 500 mutation carriers (beyond the scope of this proposal but required for confirmation of the association).

#### DNA damage response genes

The analyses of XRCC1 and XPD (also called ERCC2) were completed in year 1. The genotypes examined included XRCC1 exon 6 Arg194Trp and exon 10 Arg399Gln and XPD exon 6 C>A, 156Arg, exon 10 Asp312Asn, exon 22 C>T, Asp711, and exon 23 Lys751Gln. Three of the four XPD polymorphisms showed statistically significant association with breast cancer risk in our case population. The Lys allele at Lys751Gln in exon 23 (age-adjusted OR: 1.89; 95% CI: 1.10-3.22), the C allele (C>T, Asp711) in exon 22 (OR: 2.02; CI: 1.11-3.66) and the C allele (C>A, 156Arg) in the exon 6 (OR: 3.96; CI: 1.92-816) showed association with increased breast cancer risk in BRCA1 mutation carriers. No association between genotypes and breast cancer risk was observed for the polymorphisms in the XRCC1 gene. These data are being confirmed in the validation set as described above.

The genetic polymorphisms and population frequencies for additional DNA damage response genes have now been made publicly available by Dr. Henry Mohrenweiser at Lawrence Livermore National Laboratory and as planned, we have completed our analyses of the genes that participate in the DNA repair-related BASC complex (which also includes BRCA1) (Table 1). We studied the following variants: ATM - 5'UTR 10805 A/G, D1853N, MLH1 - 5'UTR -93 G/A, MSH2 - IVS9 -9 T/C, IVS12 -6 T/C, and MSH6 - G36E. Of the 6 variants, only the glutamine variant at codon 36 of MSH6 may be associated with the diagnosis of breast cancer (OR=2.7, 95% CI 0.86-4.9). As BRCA1 and MSH6 function in different pathways of DNA damage repair, double strand break repair and mismatch repair respectively, it is possible that alterations in multiple pathways may be more important than multiple alterations in the same pathway.

Table 1. BASC Complex polymorphisms			
Gene	Variant	Reported Frequency	Our frequency
MLH1	%'UTR -93	50%	27%
MLH1	1219V/L A>G or C	13-34%	ND
MSH2	IVS9-9T>C	20%	26%
MSH2	IVS12-6T>C	23%	10%
MSH2	G36E G>A	25%	19%
ATM	5'UTR10805 A>G	28%	55%
ATM	S49C	0.5%	2%
ATM	P1054R	1.5%	3%
ATM	D1853N G>A	25%	16%

Task 2. Perform a genome wide search to identify regions that contain novel genes, which modify breast cancer risk in BRCA1 mutation carriers (Months 18-36).

a. PCR amplification of microsatellite markers at 10-15 cM intervals throughout the genome. A request for funding for this subtask was outside the scope of this proposal and was requested from both the Center for Inherited Disease Research and The Marshfield Center. Both proposals were turned down due to what was perceived as the highly speculative nature of the project. Despite this, we believe this is an important component of the search for modifier genes, as not all may be considered a priori as candidates. Thus we undertook a directed study of chromosomes 4 and 5q, as these regions are frequently lost in BRCA1-associated breast cancers. With a limited set of relative pairs we did not see evidence of an association with age of diagnosis on chromosome 4 but found very interesting evidence of linkage on chromosome 5q. These data have recently been published and this publication is attached. The data are now being verified in the larger set of relative pairs collected as part of Task 1. Subtasks b(separation with automated sequencer gel apparatus), c (checking of all automated data analysis), d (submission of final data in linkage format output to Dr. Shugart) and e (reanalysis of samples as indicated by statistical analysis) are all described in the attached manuscript.

Task 3. Statistical analysis of data (Months 12-36)

- a. Analysis of candidate gene variants using a cohort study design based on Cox proportional hazards models and a case control design based on logistic regression analysis
- b. Analysis of linkage data using both model-based and model-free approaches. We will use both identity-by-state and identity-by-descent methods, including APM and SimIBD. This is an area of rapidly evolving methodology and improved approaches may be available within this time frame as well.

These steps are described as part of Tasks 1 and 2 and have been completed for all the genes and chromosomal regions described in those analyses.

## KEY RESEARCH ACCOMPLISHMENTS

- Collection of matched case-control set of 448 BRCA1 and BRCA2 mutation carriers (Year 1)
- Collection of a relative pair sample set containing data on 600 BRCA1 mutation carriers and DNA samples on 242 mutation carriers as components of a relative pair (Year 2)
- Completion of a comprehensive sequencing survey of immune surveillance genes for polymorphic variants (Year 1)
- Analysis of the population frequency of 26 polymorphisms in 13 immune surveillance genes (Year 1)
- Genotyping of 26 polymorphisms in 13 immune surveillance genes in the BRCA1/BRCA2 mutation carrier case-control set (Year 2)
- Identification of the immune surveillance genes TNF- $\alpha$  and IL-6 as candidate risk modifiers in the set of BRCA1/2 mutation carriers (Year 2)
- Exclusion of the immune surveillance genes IL1- $\alpha$  and - $\beta$ , IL1-RN, IL-2, IL-10, CTLA4, TNF- $\alpha$ R, IL12p35 and IL12p49 from further analysis as candidate modifier genes in this set (Year 2)
- Analysis of the population frequency of nine polymorphisms in four DNA damage response gene polymorphisms (Year 2)
- Genotyping of 16 polymorphisms in seven DNA damage response genes in the BRCA1/BRCA2 mutation carrier case-control set (Years 1 and 2)
- Identification of the DNA damage response genes TP53 and XPD (Year 1) and MSH6 (Year 2) as a candidate risk modifiers in the set of BRCA1/2 mutation carriers
- Exclusion of XRCC1 (Year 1) and ATM, MLH1 and MSH2 (Year 2) from further analysis as a risk modifier in this set of BRCA1/2 mutation carriers
- Genotyping of 25 polymorphic microsatellite repeats on chromosomes 4 and 5q at an average of 12 cM intervals in 73 BRCA1 mutation carrier relative pairs (Year 2)

- Exclusion of chromosome 4 as a locus for candidate modifier genes in this sample set (Year 2)
- Identification of chromosome 5q as a candidate region to contain a modifier of BRCA1-related breast cancer penetrance, with a maximum likelihood score at locus D5S1471 (Year 2)

## **REPORTABLE OUTCOMES**

- A manuscript is under preparation describing the immune surveillance gene polymorphism discovery and frequency evaluation. This work was presented in abstract form at the Susan B. Komen meeting in November, 2000 (abstract attached).
- A manuscript is under preparation describing the XPD and XRCC1 analyses. This work was presented in abstract form in at AACR in March, 2001 (abstract attached) and an updated, expanded analysis was presented at ASHG in October, 2001 (abstract attached).
- An abstract describing IL-6 and TNF as candidate modifiers of BRCA1 penetrance was presented at ASHG in October, 2001 (abstract attached)
- An abstract describing the analysis of the BASC complex genes as modifiers of BRCA1 penetrance was presented at ASHG in October, 2001 (abstract attached)
- A manuscript has been submitted describing the p53 polymorphism effect in women with BRCA1/2 mutations and multiple primary cancers (manuscript attached)
- A manuscript has been published describing the analysis of chromosomes 4 and 5q for candidate modifier loci (manuscript attached).
- Patents and/or licenses: None.
- Degrees obtained: None.
- Repositories, data banks and informatics tools: No new ones have been created – this work is being performed retrospectively.
- Funding applied for on the basis of this work: A pilot award from the NIH was obtained for the validation analysis of the larger set of BRCA1 and BRCA2 mutation carriers in order to confirm the positive associations described above. A full RO1 application is planned for submission October 2002.
- Employment/research opportunities: One postdoctoral fellow has completed her training with the analysis of the XRCC1 and XPD analyses and has obtained



permanent employment based on this work (Year 1). She will not be working on this project in her new position. A second postdoctoral fellow has completed her training with the work on the immune surveillance genes. She has obtained a full time faculty position in an affiliated hospital and continues to supervise work on the immune surveillance gene polymorphisms as collaboration.

## **CONCLUSIONS**

This work supports the existence of multiple genetic modifiers of BRCA1/2-related breast cancer penetrance. We have evidence that genetic variants in TNF- $\alpha$ , IL-6, p53 and XPD and MSH 6 may function in this capacity. In addition, we have evidence for a candidate locus on chromosome 5q based on a modified linkage approach. This work is important not only in ultimately leading to more refined cancer risk estimates for women with BRCA1 and BRCA2 mutations, but will also yield candidates for risk alleles in the general population as well as generate hypotheses for mechanisms that explain these effects. Once these mechanisms have been elucidated, these points in key pathways become excellent targets for preventative and therapeutic intervention.

## **REFERENCES**

None

## **APPENDIX**

Abstracts and manuscripts as described in Reportable Outcomes

**The frequency of immune surveillance gene sequence variants and association with breast cancer.**  
Anne-Marie Martin, Joel D. Greshock, Timothy R. Rebbeck and Barbara L. Weber

Breast cancer is a complex disease likely due to interactions between multiple genes and environmental influences. As many as 20% of all breast cancer patients report a family history of the disease, but only a small percentage (3-4%) of cases are directly related to germline mutations in the breast cancer susceptibility genes BRCA1 or BRCA2. While associated with as much as an 85% lifetime risk of developing breast cancer, there is both incomplete penetrance and considerable variability in the age of diagnosis in women with inherited mutations in BRCA1. Therefore it is clear that other genes play a role in defining breast cancer risk and it is likely that other genes influence the likelihood that a known carrier of a BRCA1 mutation will develop breast cancer (penetrance) as well as the age at which it is diagnosed. It has been shown that defective natural killing of tumor cells, mediated by the immune system, plays a role in preventing the clinical appearance of cancer, suggesting that variation in genes that control immune tumor surveillance could be associated with a variation in an individual's risk for cancer development. We propose that genes that control immune function could influence the penetrance of BRCA1 and that variants in these genes that have functional consequences associated with altered susceptibility to breast cancer in the general population. Therefore we have identified sequence variants in a number of genes that may play a role in tumor surveillance and we have determined the frequency of these variants in both Caucasian and African American controls. We find that there are significant racial differences in these variants. We are currently investigating the association of these newly described variants with age of diagnosis among BRCA1 carriers. This will not only provide new information about other genetic factors that may be associated with breast cancer, but may suggest new risk assessment strategies as well as generate hypothesis for biologic studies evaluating the role of these genes and associated variants in tumor surveillance.

American Association for Cancer Research, March 2001

**Role of Polymorphisms in XRCC1 and XPD as Breast Cancer Risk Modifiers in BRCA1 Mutation Carriers.**

B. Amirimani, T.R. Rebbeck, and B.L. Weber.

Department of Medicine, Center for Clinical Epidemiology and Biostatistics,  
Department of Genetics, University of Pennsylvania, Philadelphia.

Germline mutations in *BRCA1* dramatically increases lifetime risk of breast cancer. However, there is variability in the penetrance of breast cancer in *BRCA1* mutation carriers. Therefore, we hypothesize that polymorphisms in low penetrance modifier genes in the general population may play a role in modification of *BRCA1* penetrance. The association of *BRCA1* in DNA damage response suggests that *BRCA1* may maintain genomic integrity through mechanisms such as transcription-coupled repair (TCR).

We have conducted a pilot case-control study of 155 *BRCA1* mutation carrier women (103 affected with breast cancer and 52 without a breast cancer diagnosis) to evaluate the role of DNA repair genetic polymorphisms in breast cancer risk in *BRCA1* mutation carriers. The genotypes examined included six polymorphic sites in two DNA repair genes. These are XRCC1 (exon 6 Arg194Trp and exon 10 Arg399Gln) involved in base excision repair and XPD (exon 6 C>A, 156Arg, exon 10 Asp312Asn, exon 22 C>T, Asp711, and exon 23 Lys751Gln), which is active in nucleotide excision repair and TCR.

Of the polymorphisms studied, only the Gln/Gln genotype at the Lys751Gln polymorphism in exon 23 of XPD was associated with increased breast cancer risk (age-adjusted OR: 2.29; 95% CI: 1.00-5.24). No statistically significant association between genotypes and breast cancer risk was observed for any other of the polymorphisms studied here. Our results suggest that XPD may be a potential modifier risk for breast cancer in *BRCA1* carriers, but a larger sample size will be required to confirm this suggestion..

American Society of Human Genetics, October, 2001

**Polymorphisms in XRCC1 and XPD as Breast Cancer Risk Modifiers in BRCA1 Mutation Carriers.**

B. Amirimani, S.L. Neuhausen, T. Tran T.R. Rebbeck, and B.L. Weber.  
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Department of Genetics, Abramson Family Cancer  
Research Institute University of Pennsylvania, Philadelphia  
Department of Medicine, Division of Genetic Epidemiology,  
University of Utah, Salt Lake City.

BRCA1 accounts for breast cancer susceptibility in germline mutation carriers, which dramatically increase lifetime risk of breast cancer. However, there is variability in the penetrance of breast cancer in BRCA1 mutation carrier women. Therefore, we support the hypothesis that polymorphisms in low penetrance genes, called modifier genes, in the general population may play a role in alteration in defining breast cancer risk for BRCA1. The role of BRCA1 in DNA damage response suggests that other genes involved in cellular response to DNA damage are plausible candidates for low penetrance modifier genes.

In order to evaluate the role of genetic polymorphisms in DNA damage response genes in breast cancer risk in BRCA1 mutation carriers, we performed case-control analysis by genotyping 414 BRCA1 mutation carrier women (208 affected with breast cancer and 206 without a breast cancer diagnosis). The genotypes examined to date include polymorphic sites in XRCC1 (exon 6 Arg194Trp and exon 10 Arg399Gln) involved in base excision repair and XPD (exon 6 C>A, 156Arg, exon 10 Asp312Asn, exon 22 C>T, Asp711, and exon 23 Lys751Gln), which is active in nucleotide excision repair and transcription coupled repair.

Interestingly, three of four studied genotypes in XPD showed statistically significant association with breast cancer risk in our case population. Lys allele at Lys751Gln in exon 23 (age-adjusted OR: 1.89; 95% CI: 1.10-3.22), C allele (C>T, Asp711) in exon 22 (OR: 2.02; CI: 1.11-3.66) and C allele (C>A, 156Arg) in the exon 6 (OR: 3.96; CI: 1.92-8.16) showed association with increased breast cancer risk in BRCA1 mutation carriers. Odds ratios were adjusted for age at time of diagnosis and birth cohort (born before or after 1930). Whereas, no association between genotypes and breast cancer risk was observed for any of the polymorphisms in the XRCC1 gene studied here. Our results suggest that XPD may be a potential modifier risk for breast cancer in BRCA1 carriers, but exact biological effect of this association remains unclear.

**Immune surveillance genes and breast cancer: do *IL-6* or *TNF $\alpha$*  modify *BRCA1* penetrance?**

A-M. Martin, P.A. Kanetsky, G. Athanasiadis, J.D. Greshock, T.R. Rebbeck, B.L. Weber

Women who carry a mutation in the *BRCA1* gene have up to an 80% risk of developing breast cancer by the time they reach age 70. However, there is considerable variability in the age of diagnosis of breast cancer as well as incomplete penetrance in *BRCA1* mutation carriers, suggesting that other factors play a role in *BRCA1*-associated breast cancer risk. The immune system likely plays a role in preventing cancer, suggesting that variation in genes that alter control of tumor surveillance could be associated with breast cancer risk. There is evidence that cytokine gene polymorphisms are associated with different diseases, but the exact role of these polymorphisms in cancer is unknown. *IL-6* and *TNF $\alpha$*  are two good candidate genes because they play an integral role in the immune response to tumors and are highly polymorphic. We sought to determine whether polymorphisms in *IL-6* and *TNF $\alpha$*  were associated with breast cancer occurrence and age of breast cancer diagnosis among *BRCA1* mutation carriers. We studied 222 women with germline disease-associated *BRCA1* mutations, 140 (63%) of whom had breast cancer and 82 (37%) of whom had not been diagnosed with breast cancer. Although not statistically significant, we found that breast cancer cases were over twice as likely to carry two WT alleles for the TNF-238A polymorphism (OR=2.5, 95% CI: 0.88, 6.9) and were almost 50% more likely to carry any C-allele (C/\*) for the IL-6-174C polymorphism (OR=1.4, 95% CI: 0.8-2.4) compared to controls. When we stratified the IL6 analysis by age, we noted that among women 50 years of age and older, those with breast cancer were over twice as likely to have at least one C-allele (OR=2.3 95% CI: 1.0-5.1); and among younger women (<50), there did not appear to be any associated risk (OR=0.72, 95% CI: 0.3-1.7). These preliminary data suggest that that IL-6 is associated with an altered risk for breast cancer among older women, and provide the first evidence of a role for immune surveillance genes in breast cancer.

Variants in the genes that encode the BRCA1-associated genome surveillance complex (BASC) in BRCA1 mutation carriers

Katherine L. Nathanson<sup>1,2</sup>, Richard Letrero<sup>2</sup>, Peter Kanetsky<sup>3</sup>, Romaica Omaruddin<sup>2</sup>, Timothy R. Rebbeck<sup>3</sup>, Barbara L. Weber<sup>1,2</sup>

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Mutations in BRCA1 confer a greatly increased risk of breast cancer. However, the breast cancer risk due to mutations in BRCA1 varies from 40-85% depending on the population ascertained. Therefore it has been speculated that the mutation alone does not explain the observed phenotype and that other factors, including variants in genes other than BRCA1 and BRCA2, influence the development of breast cancer in BRCA1 mutation carriers. As BRCA1 and BRCA2 function in DNA damage response pathways, variants in other DNA damage response genes that are associated in complexes with BRCA1 and BRCA2 are particularly good candidate genes to modify penetrance in BRCA1 mutation carriers. We have selected variants in genes that are part of the BASC complex (MLH1, MSH2, MSH6 and ATM) as candidate genes in which single nucleotide polymorphisms may modify penetrance in BRCA1 mutation carriers. Using 221 female BRCA1 mutation carriers we studied the following variants: ATM - 5'UTR 10805 A/G, D1853N, MLH1 - 5'UTR -93 G/A, MSH2 - IVS9 -9 T/C, IVS12 -6 T/C, and MSH6 - G36E. Female BRCA1 mutation carriers were stratified by whether or not they had a breast cancer diagnosis. Of the 6 variants, only the presence of the glutamine variant at codon 36 of MSH6 may be associated with the diagnosis of breast cancer (OR=2.7, 95% CI 0.86-4.9). As BRCA1 and MSH6 function in different pathways of DNA damage repair, double strand break repair and mismatch repair respectively, it is possible that alterations in multiple pathways may be more important than multiple alterations in the same pathway.

**Germline *TP53* mutations in breast cancer families with and without multiple primary cancers: is *TP53* a modifier of *BRCA1*?**

Anne-Marie Martin<sup>1,2</sup>, Peter A. Kanetsky<sup>3</sup> Behnoosh Amirimani<sup>1,2</sup>, Theresa A. Colligon<sup>1,2</sup>, Helen A. Shih<sup>1,2</sup>, M. Renee Gerrero<sup>1,2</sup>, Kathleen Calzone<sup>4</sup>, Timothy R. Rebbeck<sup>3</sup> and Barbara L Weber<sup>1,2</sup>.

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**Keywords:**

***P53*, polymorphism, breast cancer, *BRCA1***

**Abstract:**

For women with germline *TP53* mutations who survive to adulthood, lifetime breast cancer risk may be as high as 90%. Thus *TP53* is a good candidate gene when considering the etiology of multiple primary cancers, where one is breast cancer. We studied 88 women with breast cancer and a personal or family history of multiple primary cancers (MPC), including ovarian cancer and 84 women with a personal and family history of breast cancer only (BC). All women had been previously screened for germline *BRCA1* and *BRCA2* mutations; 38 (43%) of MPC women and 10 (12%) of BC women had a mutation in one of these two genes [Shih, 2000 #3914]. We determined the frequency of deleterious germline *TP53* mutations, as well as the common R72P polymorphism in *TP53* and investigated the association of this polymorphism with the development of cancers in the entire study set. Because *BRCA1* and p53 physically and functionally interact, we also evaluated the association between R72P and breast cancer penetrance in women with known *BRCA1* or *BRCA2* mutations.

Only one woman from a family with breast cancer only had a deleterious *TP53* mutation (exon 7-G245S); no deleterious mutations were detected in the families with cases of multiple primary cancers. The common R72P polymorphism was seen at a frequency of 30.8% in the entire sample. MPC women were more likely to carry the homozygous R72 allele compared to BC women ( $p=0.02$ ; OR, 2.2, 95% CI: 1.1, 4.3). We also found that the presence of any 72P allele was associated with an earlier age of breast cancer diagnosis among *BRCA1* mutation carriers ( $p=0.02$ ) providing evidence for R72P polymorphism as a modifier of *BRCA1* penetrance.



## Introduction

Somatic alterations in *TP53* are the most frequent genetic event in human cancer and lead to inactivation of the gene, loss of tumor suppressor function and may generate a dominant-negative form of the gene product [Kern, 1992 #3886][Vogelstein, 1992 #3885][Marutani, 1999 #3883]. Eleven exons make up the primary transcript of *TP53*, of which exons 2-11 encode the protein. Five conserved domains exist in exons 1,4,5,7 and 8 [Ko, 1996 #3916], which are considered essential for normal p53 function. Approximately 90% of all disease-associated mutations occur in these domains, with mutations in five codons (175, 245, 248, 249 and 273) accounting for approximately 20% of all mutations reported to date.

Germline mutations in *TP53* cause Li-Fraumeni syndrome (LFS), a familial association of childhood leukemia, breast cancer, soft tissue sarcoma and adrenal cortical carcinoma [Li, 1969 #3509] [Li, 1982 #3917], as well as other cancers such as melanoma, germ cell tumors, and carcinomas of the lung, pancreas, and prostate [Strong, 1987 #1421] [Li, 1988 #3508]. Cancers characteristically develop at unusually early ages, and multiple primary tumors are frequent. Susceptibility to cancer in these families follows an autosomal dominant pattern of inheritance [Strong, 1987 #1421] and among families with a known germline *TP53* mutation the probability of developing any invasive cancer (excluding carcinomas of the skin) approaches almost 50% by age 30, compared to an age-adjusted population incidence of cancer of 1%. It is estimated that more than 90% of *TP53* mutation carriers will develop cancer by age 70 [Malkin, 1990 #1937].

In families with an extensive history of cancer that do not fit the criteria for LFS, the frequency of *TP53* germline mutations is not well defined. Borresen et al. (1992) have demonstrated that germline *TP53* mutations account for <1% of site-specific breast cancer families [Borresen, 1992 #3413], however, among LFS families, there is a very high incidence of early onset breast cancer. Taken together, these data suggest that *TP53* contributes to hereditary breast cancer susceptibility in the context of LFS but is a very rare cause of breast cancer in the general population.

Because of the high penetrance of early onset breast cancer and the known increased incidence of multiple primary cancers in LFS families (50% by 30 years of age) (reviewed in Malkin et al., 1990) we investigated whether deleterious germline mutations in *TP53* and/or the R72P polymorphism were associated with multiple primary cancers in families with multiple primary cancers in which one was breast cancer. To this end we determined the frequency of deleterious *TP53* germline mutations in a sample of 172 breast cancer families with and without multiple primary cancers with known *BRCA1* and *BRCA2* mutation status (43% of women with multiple primary cancers had a germline *BRCA1* or *BRCA2* mutation compared to 12% of women with breast cancer only). Furthermore, we established the frequency of the common exon 4 polymorphism (R72P) in this sample and evaluated whether this polymorphism may be a modifier of breast cancer penetrance.

## Materials and Methods

### *Patient Population*

All families were recruited from clinics at the University of Michigan (1993-1995) and the University of Pennsylvania (1995-1998). Patients were either self- or physician referred because of a perceived elevated risk of inherited susceptibility to breast cancer. All women consented genetic testing for clinical and/or research purposes. Personal and family histories of all cancers were recorded, including age of diagnosis of all cancers and the number of related women in each family at risk for breast cancer (age  $\geq 20$  years). Pathology reports were obtained on all probands and on other family members when possible. The testing protocol was approved by duly constituted institutional review boards at both the University of Michigan and the University of Pennsylvania.

Eighty-eight women were from families with at least two cases of breast cancer and at least one woman affected with both a primary breast cancer and a primary non-breast cancer; (denoted MPC). All non-breast malignancies were considered, including non-melanoma skin cancers. Seventy-seven (88.8%) MPC women had two primary cancers, and 11 MPC women (11.2%) had three or more primary cancers. The remaining 84 women were from families with at least two cases of breast cancer but no cases of multiple primary cancers; (denoted BC). All samples were previously screened for germline mutations in *BRCA1* and *BRCA2* [Shih, 2000 #3914]. Of the samples tested, 38 MPC women had a *BRCA1* or *BRCA2* germline mutation compared to 10 BC women [Shih, 2000 #3914].

### ***Mutation analysis:***

DNA was extracted from peripheral blood mononuclear cells and stored in TE at 4°C. The entire 10-exon coding domain and flanking splice site regions of *TP53* were amplified using 7 PCR primer sets (table 1). PCR amplification was performed in a final volume of 20µl containing 80ng of DNA, 1.5mM of MgCl<sub>2</sub>, 10mM Tris-HCl (pH 8.3), 50mM KCl, 0.2mM each of dCTP, dATP, dTTP, dGTP (Amersham Pharmacia Biotech), each primer at 1.0 µM and 1.0 unit of Taq polymerase (Boehringer Mannheim). Annealing temperatures were optimized for each primer set and ranged from 55-60°C. Variants were identified by conformation sensitive gel electrophoresis (CSGE) as previously described [Shih, 2000 #3914] and characterized by direct sequencing using the ABI prism 377 after reamplification from source DNA.

### ***Statistical analysis:***

Differences in *TP53* mutation frequency between MPC and BC groups were assessed using  $\chi^2$  analysis or Fisher's exact test when necessary. We used the Mann-Whitney U-Wilcoxon rank sum test to determine whether *TP53* genotypes altered the median age of first breast cancer diagnosis within categories defined by *BRCA1* or *BRCA2* mutation status.

### **Results:**

We determined the *TP53* mutation status using CSGE analysis and direct sequencing. DNA from one woman from a BC family and no *BRCA1* or *BRCA2* mutation showed an abnormal CSGE profile in exon 7. Sequence analysis of this variant revealed a G to A transition at the first nucleotide in codon 245 resulting in a glycine ->

serine change at this position (G245S). No deleterious *TP53* mutations were seen in the MPC group [MPC = 0%, BC = 1.2%,  $p=0.31$ ].

The proline allele of the R72P polymorphism was seen at a frequency of 30.8% in the entire sample. The homozygous R72 genotype occurred at a greater frequency among MPC women (77%) as compared to BC women (61%) ( $p=0.02$ ; OR, 2.2, 95% CI: 1.1, 4.3) (Table 2). When we performed subgroup analyses in women with a *BRCA1* or *BRCA2* mutation, MPC women were four times more likely to have the homozygous R72 genotype than BC women (OR=4.1, 95% CI: 1.2, 14.3) (Table 3). This association between the homozygous R72 genotype and MPC was most striking in women with *BRCA1* mutations (OR=5.1, 95% CI: 0.89,29.3), but possibly due to a smaller sample size, neither the analysis of the *BRCA1* nor the *BRCA2* subset separately revealed a significant association.

In an evaluation of the R72P polymorphism as a modifier of breast cancer penetrance in women with germline *BRCA1* or *BRCA2* mutations, we found that among *BRCA1* mutation carriers, the presence of any 72P allele was associated with an earlier median age of breast cancer diagnosis (median age=33, interquartile range (IQR) 29-41) compared with the homozygous R72 genotype (median age=44.5, IQR 36-51;  $p<0.02$ ) (Table 4). This association was not seen in *BRCA2* mutation carriers, or in the combined *BRCA1* and *BRCA2* mutation carrier analysis.

## Discussion:

In this study, we screened all 10 coding exons of the *TP53* gene in women with a personal or family history of breast cancer with or without multiple primary cancers that also had been characterized for *BRCA1* and *BRCA2* mutations. We identified one presumably deleterious missense mutation (G245S) in an individual from a family with a history of site-specific breast cancer only. This patient did not carry a germline mutation in either *BRCA1* or *BRCA2* [Shih, 2000 #3914]. The G245S missense mutation has been reported previously in the germline of a woman with breast cancer [Borresen, 1992 #3413] and the germline of a man with sarcoma [Toguchida, 1992 #1377]. Consistent with the work of Borresen *et al*, 1992, in our study the proband was diagnosed with breast cancer at the age of 29. In addition, her sister was diagnosed with breast cancer at the age of 27 and went on to develop a second primary breast cancer at 31 years of age (Figure 1). Of interest however, the G245S mutation was not detected in DNA from the sister's first breast tumor (no germline DNA was available). In addition, there was no allelic loss of flanking *TP53* in that tumor (data not shown), suggesting that either the proband's sister is a phenocopy or the *TP53* mutation is not the relevant source of breast cancer susceptibility in this family. Thus, we conclude that germline *TP53* mutations are not an important cause of multiple primary cancers in families with a history of breast cancer and provide support for previous studies suggesting that germline *TP53* mutations are an infrequent cause of familial site-specific breast cancer.

In the present study the R72P polymorphism was seen at a frequency of 30.8% in the entire sample and we observed a four-fold higher frequency of the homozygous R72

genotype among MPC women with *BRCA1* or *BRCA2* mutations compared to BC women with a *BRCA1* or *BRCA2* mutation. These data suggest that women who are homozygous for the R72 allele and have a mutation in *BRCA1* or *BRCA2* may be at increased risk for developing multiple primary cancers.

Other studies associating the homozygous R72 allele and increased cancer risk have been reported. For instance, the association between *TP53* polymorphisms and human papillomavirus (HPV)-associated cervical cancer has been examined. In 1998, Storey *et al*, suggested that women who were homozygous for the R72 allele were seven times more susceptible to HPV-related cervical cancer than women who were heterozygotes [Storey, 1998 #4520]. However, these data have been difficult to replicate and an equal number of studies have either confirmed [Makni, 2000 #4545][Agorastos, 2000 #4544][Dokianakis, 2000 #4546] or disputed [Tenti, 2000 #4548][Tachezy, 1999 #4549][Yamashita, 1999 #4551][Klaes, 1999 #4553][Rosenthal, 1998 #4559] the R72 association with cervical cancer.

In the subset analysis of the R72P polymorphism as a candidate modifier of breast cancer penetrance in *BRCA1/2* mutation carriers, we observed that the presence of any proline allele was significantly associated with an earlier age of breast cancer diagnosis among women with a *BRCA1* mutation. One possible explanation for the association of the proline allele with earlier onset breast cancer in *BRCA1* mutation carriers and the association of the arginine allele with MPC could be that there is an excess or earlier mortality among women with an earlier age of diagnosis of breast cancer, (i.e. those with

the proline allele). Thus women homozygous for the arginine allele may live longer and have a greater likelihood of developing a second cancer.

BRCA1 and p53 (but not BRCA2) physically interact with one another *in vitro* and *in vivo* resulting in enhanced p53-mediated transcription [Ouchi, 1998 #259] [Zhang, 1998 #628]. There are two p53 binding sites in BRCA1; one is close to the nuclear localization signal in the N-terminal region of exon 11 [Zhang, 1998 #628] and one is in the most C-terminal BRCT domain [Chai, 1999 #3737]. Deletion of the N-terminal exon 11 p53-binding site prevents *in vitro* interaction of the two proteins and abrogates the BRCA1-p53 responsive promoters such as bax, p21 and GADD45 [Zhang, 1998 #628] [Harkin, 1999 #3754]. In addition, a truncation mutant of BRCA1 that retains the p53-interacting site but removes the C-terminal BRCA1 transactivation domain acts as a dominant inhibitor of p53-dependent transcription [Zhang, 1998 #628]. Finally *TP53* mutations may be more common in *BRCA1*-associated breast cancers than sporadic or *BRCA2*-associated tumors. It has been reported that *TP53* somatic mutations are found in greater than 80% of *BRCA1*-associated tumors [Crook, 1997 #420] and reviewed in [Schuyer, 1999 #3921]. These data have led to the speculation that *TP53* mutations, or another component of the relevant pathway, maybe required before *BRCA1*-related tumorigenesis can proceed [Bertwistle, 1998 #100]. Recent data from murine models supports this hypothesis [Xu, 2001 #3922].

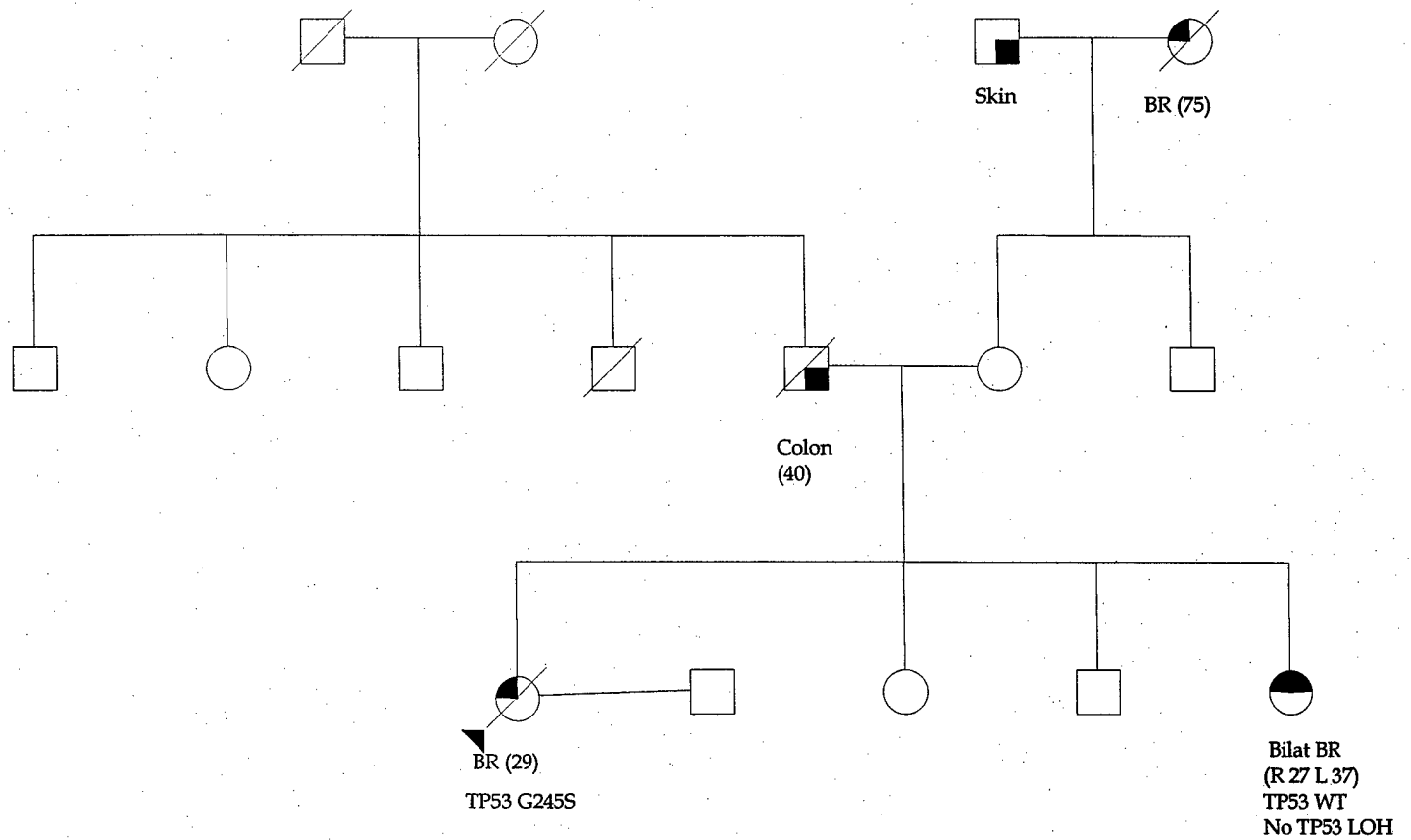
These data suggest a critical role for the p53/BRCA1 interaction, providing a possible explanation for the association of *TP53* variants and cancer risk in women with



*BRCA1* mutations, as well as a possible explanation for why this association was not seen in *BRCA2* mutation carriers. Thus, it is possible that the R72P polymorphism in *TP53* alters the p53/BRCA1 interaction and in turn alters BRCA1-associated tumorigenesis.

In summary, we provide evidence that germline mutations in *TP53* are not associated with hereditary susceptibility to site-specific breast cancer or with the presence of multiple primary cancers in breast cancer families, supporting previous studies that *TP53* mutations account for less than 1% of hereditary susceptibility to breast cancer. However, we found presence of the homozygous R72 allele was associated with the development of multiple primary cancers among individuals with a germline *BRCA1* or *BRCA2* mutation. Finally, we provide the first evidence that the arginine allele of R72P in exon 4 of *TP53* may modify *BRCA1*-associated breast cancer risk, using age of diagnosis as a surrogate for penetrance.

Figure 1: Pedigree of patient with germline *TP53* mutation. Numbers in parentheses indicate age of cancer diagnosis.



**Table 1: Primer Sequences**

Exon	Primer sequences	Annealing Temp. (°C)	Reference
2/3	Forward: 5'-ggatccccacttttctctt-3' Reverse: 5'-agcatcaaatcatccattgc-3'	57	
4	Forward: 5'-gacctggctcctctgactgct-3' Reverse: 5'-atacggccaggcattgaagt-3'	54	
5/6	Forward: 5'-tgccctgactttcaactctgt-3' Reverse: 5'-ttaaccctcctcccagaga-3'	54	
7	Forward: 5'-tgccacaggtctccccaagg-3' Reverse: 5'-aggggtcagcggcaagcaga-3'	55	Evans et al. 1998
8/9	Forward: 5'-caagggtggtgggagtaga-3' Reverse: 5'-acttgataagaggtccaag-3'	54	
10	Forward: 5'-atgttgctttgatccgtca-3' Reverse: 5'-ctttccaacctaggaaggca-3'	54	
11	Forward: 5'-agccacctaactcaaaaa-3' Reverse: 5'-aatggcaggggaggagaga-3'	55	Evans et al. 1998

**Table 2: The frequency of the *TP53* exon 4 R72P polymorphism in individuals with multiple primary cancers (MPC) compared to individuals with breast cancer only (BC)**

<i>TP53</i> Codon 72 Genotype	MPC n=88		BC n=84		OR	95% CI	p value
	n	(%)	n	(%)			
R/R	68	(77)	51	(61)	2.2	(1.1, 4.3)	0.02
R/P	17	(19)	28	(33)	1.0	(0.21, 4.8)	
P/P	3	(3)	5	(6)	1.0		

**Table 3: *TP53* R72P allele frequencies in MPC and BC families**

<i>TP53</i> Exon 4 Codon 72 Genotype	MPC n=88		BC n=84		OR	95% CI
	n	(%)	n	(%)		
BRCA1 or 2 Mutation						
R/R	29	(76)	7	(44)	4.1	(1.2, 14.3)
P/*	9	(24)	9	(56)	1.0	
BRCA1 Mutation						
R/R	23	(79)	3	(43)	5.1	(0.89, 29.3)
P/*	6	(21)	4	(57)	1.0	
BRCA2 Mutation						
R/R	8	(67)	4	(44)	2.5	(0.42, 14.8)
P/*	4	(33)	5	(56)	1.0	
No Detectable Mutation						
R/R	39	(78)	44	(65)	1.9	(0.84, 4.5)
P/*	11	(22)	24	(35)	1.0	

N.B. P/\* = R72P or P72P

**Table 4: *TP53* R72P allele frequencies and age of diagnosis of breast cancer**

<i>TP53</i> Codon 72 Genotype	Median Age	(IQR)	p-value
BRCA1 or 2 Mutation			
R/R (n=36)	42	(36-53.5)	.07
P/* (n=18)	35	(30-45)	
BRCA1 Mutation			
R/R (n=26)	44.5	(36-51)	.02
P/* (n=10)	32	(29-41)	
BRCA2 Mutation			
R/R (n=12)	40.5	(36.5-58)	.97
P/* (n=9)	43	(34-64)	
No Detectable mutation			
R/R (n=83)	49	(39-61)	.94
P/* (n=35)	49	(42-57)	

# CGH-targeted linkage analysis reveals a possible *BRCA1* modifier locus on chromosome 5q

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Women with germline mutations in *BRCA1* have a greatly elevated risk of breast and ovarian cancer. However, considerable variation in the degree of breast cancer risk associated with a *BRCA1* mutation has been observed, suggesting that modifiers of *BRCA1* penetrance may exist. We hypothesized that the modifier genes might be located in regions of allelic imbalance in the tumors of *BRCA1* mutation carriers, as have been reported on chromosomes 4p, 4q and 5q. In order to determine whether novel genetic modifiers of *BRCA1*-associated breast cancer penetrance in these regions exist, we used non-parametric linkage analysis methods to determine whether allele sharing of chromosomes 4p, 4q and 5q was observed preferentially within *BRCA1* mutation families in women with *BRCA1* mutations and breast cancer. No significant linkage on chromosome 4p or 4q was observed associated with breast cancer risk in *BRCA1* mutation carriers. However, we observed a significant linkage signal at D5S1471 on chromosome 5q ( $P=0.009$ ) in all the families analyzed together. The significance of this observation increased in the subset of families with an average of breast cancer diagnosis less than 45 years ( $P=0.003$ ). These results suggest the presence of one or more genes on chromosome 5q33–34 that modify breast cancer risk in *BRCA1* mutation carriers. The approach described here may be utilized to identify penetrance modifiers in other autosomal dominant syndromes.

## INTRODUCTION

The observation of substantial variability in penetrance of breast cancer due to germline mutations in *BRCA1* has led to speculation that inheritance of a *BRCA1* mutation alone may not completely describe the observed cancer phenotype. The initial estimate of *BRCA1*-associated breast cancer risk of 85% was calculated using the original families ascertained for *BRCA1* linkage analysis. However, population- and hospital-based studies have estimated a lifetime breast cancer risk in *BRCA1* mutation carriers of 36–56% (1–6). In a recent hospital-based study of Ashkenazi Jewish women, the risk of breast cancer due to the two Ashkenazi Jewish *BRCA1* founder mutations was estimated to be 46%, substantiating the lower breast cancer risk seen in previous studies (7). Thus breast cancer risk due to *BRCA1* mutation differs based on the population of ascertainment, suggesting that other factors – either genetic or environmental – modify breast cancer risk.

While germline mutations in *BRCA1* and *BRCA2* account for the majority of families with both breast and ovarian cancers, they account for only a minority of families with multiple cases of breast cancer only. Studies in populations of early-onset breast cancer cases have demonstrated that approximately 85% of the breast cancer risk to mothers and sisters is not attributable to mutations in either gene, suggesting the presence of variants in additional genes that confer breast cancer risk (8–10). While mutations or variants in susceptibility genes other than *BRCA1* or *BRCA2* could presumably act alone to affect breast cancer risk in the general population, in *BRCA1* mutation carriers they could act in concert with the high-penetrance mutation to modify age at breast cancer diagnosis. In fact, using cases of breast cancer under the age of 55 years from the East Anglia Cancer Registry (UK), after accounting for mutations in *BRCA1* and *BRCA2* and adjusting for parity, a polygenic model was the best explanation for additional breast cancer susceptibility genes. In addition, these putative common susceptibility alleles have an effect in both *BRCA1* and *BRCA2*

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mutation carriers and those without mutations (11). Therefore, the evidence suggests that genetic variation, other than that due to mutations in BRCA1 and BRCA2, contributes to breast cancer susceptibility.

Several approaches have been taken to identify additional breast cancer susceptibility genes. These approaches have included linkage studies, population-based case-control association studies, and case-control association studies in BRCA1 and BRCA2 mutation carriers using those affected with cancer as cases and those without cancer as controls. This third group of studies mainly has examined candidate modifier genes, selected on the basis of biological plausibility (12–14). The first such study demonstrated an association of rare alleles of HRAS with ovarian cancer (a 2.11-fold risk increase) but not of breast cancer (15). Additional studies have demonstrated that increased length of CAG repeats within both the androgen receptor (AR, Xq11.2–12) and AIB1 (20q12) are significantly associated with increased breast cancer risk in BRCA1 mutation carriers (16,17). However, the AR genotypes associated with increased risk are infrequent in the population and unlikely to account for all of the phenotypic variability. In addition, replication studies have not shown consistent results (18,19). Association studies using variants in candidate genes support the evidence that genetic modifiers of penetrance in BRCA1 and BRCA2 mutation carriers exist.

While variants in candidate genes have been associated with altered penetrance in BRCA1 mutation carriers, these studies are intrinsically limited to known genes selected on the basis of biological plausibility. Therefore, we sought to determine whether chromosomal regions containing yet-uncharacterized genes that alter breast cancer risk in BRCA1 mutation carriers could be identified. In order to prioritize chromosomal regions likely to contain genes affecting breast cancer penetrance, we used data from comparative genome hybridization (CGH) studies in BRCA1 mutation carriers. CGH is sensitive to both chromosomal gains and losses, indicative of the presence of oncogenes and tumor suppressors, respectively. The most consistently observed alterations in breast tumors from BRCA1 mutation carriers were loss of the chromosomal arms 5q (86%), 4q22-qter (81%) and 4p (64%). In contrast, at 5q, 4q and 4p, sporadic breast tumors show losses of 11% (20,21). While many modifiers of penetrance are presumably not tumor suppressor genes that select for deletions, consistent loss of 4p, 4q and 5q provides the strongest available evidence that there are genes in these regions related to BRCA1-associated tumorigenesis.

## RESULTS

For the initial evaluation of chromosomes 4 and 5q, microsatellite markers spaced at an average of 12 cM across these regions were analyzed in 18 families (Fig. 1). Twelve families had deleterious BRCA1 mutations. Six families had early-onset breast cancer co-segregating with polymorphic markers on 17q21 surrounding BRCA1, but no detectable mutations. In order to maximize the potential of finding a modifier locus in this initial evaluation, we were as inclusive of families as possible. The initial analysis was performed using identity-by-descent methods without assuming a model of

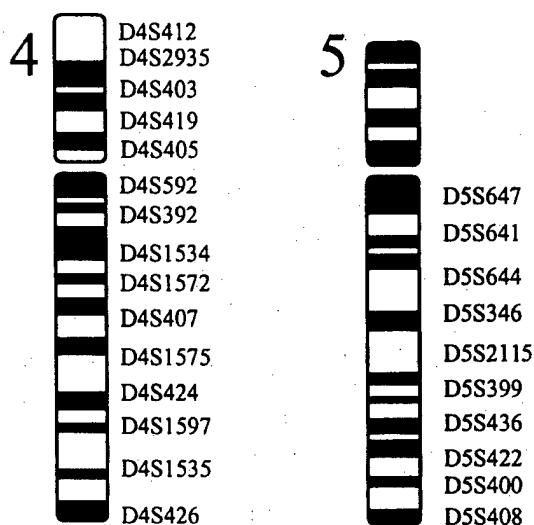


Figure 1. The markers used in the initial screen for modifiers of penetrance in BRCA1 mutation carriers are shown across chromosomes 4 and 5q.

inheritance for a modifier gene effect. Using SimIBD, we did not identify any significant candidate loci associated with breast cancer in BRCA1 mutation carriers on chromosome 4. However, we identified 3 markers on chromosome 5q with P-values less than 0.05: D5S495 ( $P=0.016$ ), D5S2115 ( $P=0.009$ ) and D5S400 ( $P=0.02$ ).

In order to further explore the possibility that one or more modifier genes exist at these loci, we increased both the stringency of the family selection for BRCA1 mutation carriers and the marker density at the loci with  $P < 0.05$ . We added four newly identified families with known deleterious mutations in BRCA1 and removed four of the six families with evidence of linkage to 17q and posterior probabilities of carrying a mutation in BRCA1 less than 90%, for 18 families in total. The remaining two families without detectable mutations in BRCA1 had posterior probabilities of carrying a mutation in BRCA1 greater than 90% (96% and 98%). The families with posterior probabilities less than 90% were eliminated so that the population studied was as homogenous as possible in the second stage of analysis. Additional markers were targeted to the locations of the initial positive SimIBD scores, so that fine mapping could be performed (Fig. 2). In order to perform multiple analyses with all the families, including those with extended pedigrees, SimWalk2 was used, and the statistic E (equivalent to  $S_{all}$  in Genhunter) was calculated (22).

After the families were analyzed in total, they were stratified in two different groups. In the first stratified analysis, the families were classified by average age of breast cancer diagnosis. Women diagnosed with ovarian cancer only or with ovarian cancer prior to their breast cancer diagnosis were not included either in the calculations or in the analysis, since their therapy for ovarian cancer may have affected their risk of breast cancer. In addition, the families were grouped by the number of breast cancer cases per family ( $< 6$ , 7 families or  $\geq 6$ , 11 families).

When all the families were analyzed together, the strongest linkage signal was seen at D5S1471 ( $P=0.009$ ) (Fig. 2).

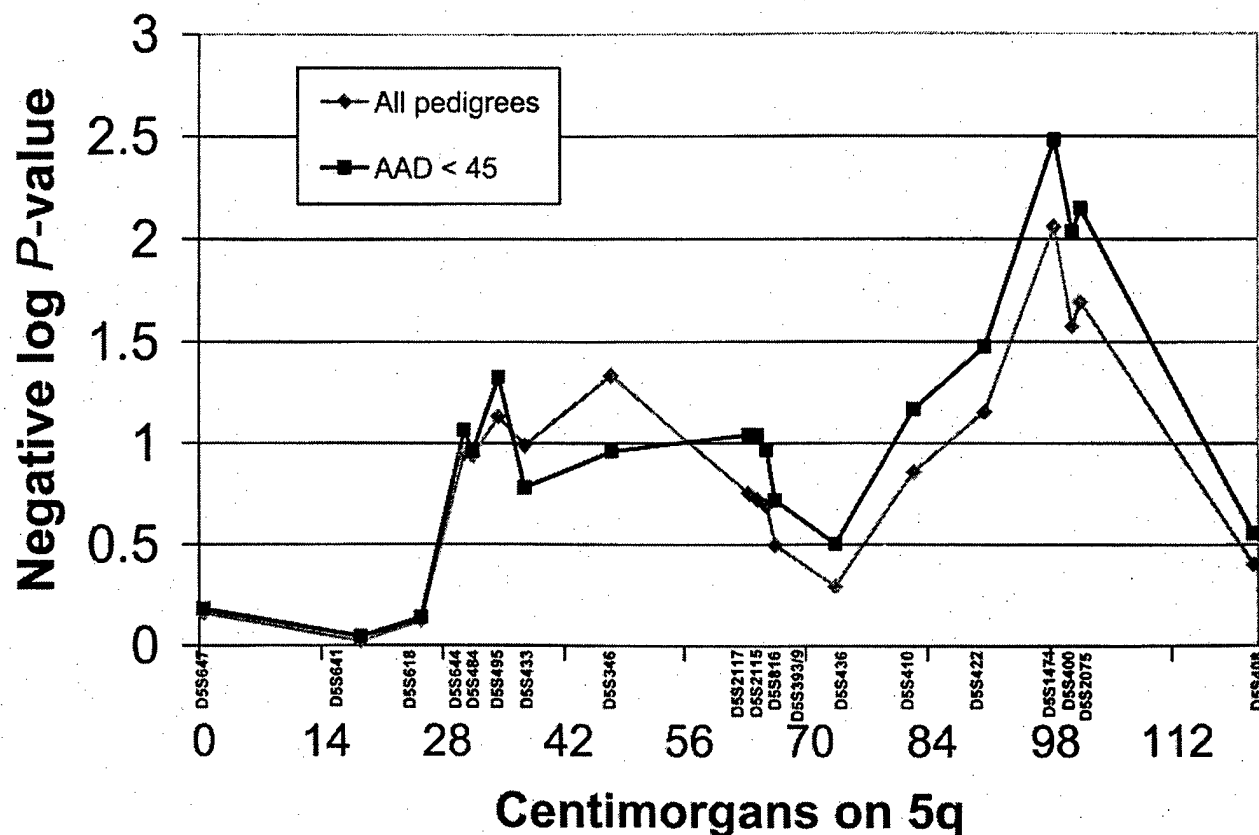


Figure 2. The results of the second stage of analysis looking for modifiers of penetrance in BRCA1 mutation carriers using SimWalk2 are shown as negative logarithm of the P-values obtained, with the markers used shown on the x-axis. The marker distance is shown in centimorgans across 5q, with D5S647 (centromeric marker) at 0 cM and D5S408 (telomeric marker) at 121 cM. Results are demonstrated as a negative log P values for greater discrimination. All pedigrees: The SimWalk2 analysis results for all families together, showing the peak negative logarithm of the P-value 2.06 at D5S1471, corresponding to a P-value of 0.009. AAD < 45: The SimWalk2 analysis results in families with an average age of breast cancer diagnosis 45 years or less, showing the peak negative logarithm of the P-value at 2.48, corresponding to a P-value of 0.003.

This effect was even more pronounced in families with an average age of diagnosis of 45 years or less ( $n=15$ ) ( $P=0.003$ ). No difference in P-values was seen when the families were stratified by number of breast cancer cases per family (data not shown).

## DISCUSSION

These results suggest that gene or genes located on chromosome 5q33–34 may modify breast cancer penetrance in kindreds with germline BRCA1 mutations. Given that the most significant results were seen in families with an average age of diagnosis less than 45 years, we postulate that a variant in a gene or genes within that region increases the age-adjusted penetrance of BRCA1 mutations. The region within which the gene(s) is most likely located in the interval defined by an 10-fold increase in the P-value from the peak of 0.003 to 0.03. This interval encompasses from D5S422 to approximately halfway between D5S2075 and D5S408, 10 Mb centered around D5S1471.

In order to make a preliminary list of genes within this region that may be responsible for the effects observed here, we compared the set of known and predicted genes (hypothetical proteins) that were localized by sequence to this region in three databases: the Human Genome sequence (NCBI Build 27), Ensembl (v1.2.0) and the Celera database (v3.3). For this analysis, we included known genes and novel transcripts of more than one exon when there was additional supporting evidence for their existence from other sources such as Unigene or EMBL. We then compared the lists of genes from each source, and eliminated known or predicted genes that were only localized to this region by one of the three data sources (Table 1). Within this region, the Ensembl and Celera databases were very consistent. However, the NCBI region contained a contig (NT\_006907.7) that had been within this region on earlier builds, but was now located approximately 10 cM proximal to the region. As the markers within the contig are consistent with its location within the 10 Mb region of interest, the genes on the contig are considered in Table 1. Although the genome annotation is unfinished in this region, this list of genes and transcripts provides a starting point that can be expanded as the

Table 1. Genes located in the 10 Mb region around D5S1471: all known or predicted genes in two of the three databases examined are included

Gene	NCBI	Celera	Ensembl	Protein
CCNG1	X	X	X	Cyclin G1
DOCK2	X	X	X	Dedicator of cytokinesis 2
FBXW1B	X	X	X	f-box and WD-40 domain protein 1B
FGF18	X	X	X	Fibroblast growth factor 18
FLJ12899	O	X	X	Hypothetical protein similar to panthothenate kinase 1 $\beta$
FLJ20364	—	X	X	Hypothetical protein/arsenite-related gene 1
FOXI1	X	X	X	Forkhead box I1
GABRP	X	X	X	GABA receptor $\pi$
HMMR	X	X	X	Hyaluronan-mediated motility receptor
KCNIP1	X	X	X	Kv channel-interacting protein
KCNMB1	X	X	X	K <sup>+</sup> large conductance Ca <sup>2+</sup> -activated channel, M $\beta$ 1
LCP2	X	X	X	Lymphocyte cytosolic protein 2
KIAA0869	O	X	X	Q94946, XM_047992
LOC96317	X	—	X	Similar to ubiquitin carboxyl-terminal hydrolase
LOC116275/6	X	—	X	Similar to fucosyltransferase 5
LOC116280	X	—	X	Similar to neuritin precursor
LOC86683	X	X	X	Similar to 60S ribosomal protein L10
MAT2B	X	X	X	Methionine adenosyltransferase II, $\beta$
NPM1	X	X	8 <sup>a</sup>	Nucleophosmin
ODZ2	O	X	X	Odd Oz/ten-m homolog 2
RANBP17	X	X	X	RAN-binding protein 17
RARS	O	X	X	Arginyl-tRNA synthetase
SLIT3	O	X	X	Slit homolog 3
STK10	X	X	X	Serine threonine kinase 10
TLX3	X	X	X	Homeobox 11-like2

X, known or predicted genes within the region represented on 2 or more of the databases; O, the genes on NT\_006907.7, which was in the region on earlier NCBI builds and contains markers consistent with its location within the region; —, the known or predicted gene is not represented in database examined.

<sup>a</sup>NPM1 is located on chromosome 8 in Ensembl.

annotation is completed. Of the genes within the region, cyclin G1 (CCNG1) is of particular interest, since it is a transcriptional target of p53, which is known to interact with BRCA1, localizes to nuclear foci after DNA damage, and is upregulated in breast involution (23,24).

While there are interesting candidate genes in the region identified, further studies are necessary to confirm our finding. The data suggest that a genome-wide search to identify modifiers using this approach in a larger number of families with BRCA1 mutations is feasible. In addition to being able to replicate these findings in a larger sample set, we shall be able to subset families into more genetically homogenous groups, based on mutation status and ethnicity, such as the Ashkenazi Jewish population. Genetically homogenous study groups also may allow us to localize modifiers that are population-specific. Our data also suggest that allele-sharing methods may be useful in identifying loci of modifier genes that interact with mutations detected in autosomal dominant familial cancer syndromes in which mutation status in part determines the phenotype of interest (e.g. cancer penetrance). This approach circumvents the limitations of the candidate gene approach, and could be applied in a wide variety of autosomal dominant syndromes in which variable penetrance has been demonstrated and an underlying genetic cause has been postulated but not as yet elucidated, such as neurofibromatosis type 1 and von Hippel-Lindau disease (25,26). Novel genes identified that alter cancer susceptibility in the syndromic setting also may be considered candidate low-penetrance cancer susceptibility genes in the general population and then evaluated in larger case-control studies.

## MATERIALS AND METHODS

### Patients

BRCA1 mutation carriers were ascertained through families recruited for research studies or the Cancer Risk Evaluation Program at the University of Pennsylvania. This study was reviewed and approved by the Institutional Review Board of the University of Pennsylvania.

For the initial screen, 18 families were analyzed: 12 with BRCA1 mutations and 6 with evidence of linkage to BRCA1 but no detectable mutations, with early-onset breast cancer co-segregating with polymorphic markers on 17q21. The posterior probabilities for the latter set of carrying a BRCA1 mutation ranged from 20% to 98%. Women were considered as affecteds for linkage purposes if they were affected with breast cancer and were carriers of the deleterious BRCA1 mutation or the familial linked haplotype. Seventy-three women with breast cancer were genotyped: 44 carried a deleterious mutation in BRCA1 and 29 carried the 17q disease-associated haplotype in their family.

In the latter stage of this study (fine mapping on chromosome 5q and more stringent familial selection), 16 families with deleterious mutations and 2 families with a posterior probability greater than 90% of being due to mutations in BRCA1, but no detectable mutation, were analyzed. The 16 families with deleterious BRCA1 mutations contained the 12 families in the initial analysis and 4 additional newly identified families. Fifty-five female BRCA1 mutation carriers and 9 women who shared 17q haplotypes were typed in the



18 families. Within these families, the women with breast cancer and BRCA1 mutations or linkage to the shared 17q haplotype were contained within 22 sib pairs, 20 second-degree relative pairs, 22 third-degree relative pairs and 18 fourth-degree or greater relative pairs, for 82 relative pairs in total. So that the identity-by-descent methods could be utilized to their full advantage, unaffected parents and siblings, female and male, were genotyped where available. In total, 70 relatives without breast cancer from 15 families, those with relatives available, were typed, ranging from 2 to 8 per family. These included 38 males (13 with and 25 without BRCA1 mutations) and 32 females (5 with and 27 without BRCA1 mutations). The 5 females with BRCA1 mutations were either unaffected and under age 25 years or affected with ovarian cancer. The 16 families with disease-associated mutations in BRCA1 contained a range of mutations, including missense (C64Y), nonsense (E143X, Y1563X), frameshift (185delAG  $n=3$ , 2187delA, 2274insA, 2800delA, 3875del4, 4286delTG, 5382insC, 5438insC) and splice site (IVS8 + 2 T > A) mutations, as well as genomic rearrangements (intron 7-9 deletion, exon 13 duplication).

#### Posterior probability of linkage to BRCA1

Posterior probabilities of disease being due to a mutation in BRCA1 were calculated for the seven families that showed linkage to 17q21 without a deleterious mutation. The prior probability of breast cancer susceptibility being due to BRCA1 was assumed to be 0.8 for breast-ovarian cancer families and 0.3 for site-specific breast cancer families (27). A sequential application of Bayes' rule incorporating lod scores and subsequently the mutation screening data, with detection sensitivities of 0.7 for CSGE and 0.85 for CSGE/Southern blotting, was used to calculate the posterior probabilities.

#### Markers

Genomic DNA was purified from peripheral lymphocytes and 60 ng per reaction was amplified by PCR in 15  $\mu$ l containing 0.33  $\mu$ M primer, 25  $\mu$ M each dNTP, 1  $\times$  PCR buffer II (Perkin-Elmer) and 0.6 U AmpliTaq Gold (Perkin-Elmer). PCR cycles were as follows: 95°C for 12 min, then 10 cycles at 94°C for 15 s, annealing at 55°C for 15 s and extension at 72°C for 15 s, followed by 20 cycles at 89°C for 15 s, annealing at 55°C, and extension at 72°C with a final extension at 72°C for 10 min. Primers were fluorescently labeled and products were separated on 4% polyacrylamide gels, using an ABI Prism 377 automated sequencer, and genotypes were identified by use of ABI Genescan/Genotyper software (PE Biosystems). All genotypes were checked for Mendelian inheritance, both by inspection and by use of PEDCHECK (28). Attempts were made to resolve all inconsistent genotypes by repeating the PCR amplification and rerunning the laboratory analyses. If a genotype remained inconsistent after these checks, it was removed from the analysis. In total, 0.7% of the genotypes were removed from the analysis.

#### Linkage methods

In the absence of a clear understanding of the mode of inheritance of the trait of interest, we used 'model-free' methods for both single- and multiple-marker analyses. Single-marker analysis was performed with the SimIBD developed by Davis et al. (29), which is ideal for analyzing data sets that contain multiple pairs of affected relatives. SimIBD is based on a simulation-based IBD statistic, and uses a recursive algorithm to determine the probability of two affecteds sharing a specific allele IBD. Therefore the method is suitable for analyzing the multiplex pedigrees that we have collected. For fine mapping on chromosome 5q, SimWalk2 (v2.6) was used, and the statistic E [equivalent to the  $S_{all}$  in Genehunter (v2.1)] was calculated in order to perform multiple analyses with all the families, including those with extended pedigrees (22), since the current version of SimIBD cannot be used for multipoint analysis and the Genehunter program has limitation on pedigree size (30). Marker allele frequencies were calculated using data from one individual randomly chosen from each pedigree.

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#### REFERENCES

1. Ford, D., Easton, D.F., Bishop, D.T., Narod, S.A. and Goldgar, D.E. (1994) Risks of cancer in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. *Lancet*, 343, 692-695.
2. Easton, D.F., Bishop, D.T., Ford, D., Crockford, G.P. and Consortium, B.C.L. (1994) Breast and ovarian cancer incidence in BRCA1 mutation carriers. *Lancet*, 343, 962.
3. Narod, S.A., Ford, D., Devilee, P., Barkardottir, R.B., Lynch, H.T., Smith, S.A., Ponder, B.A., Weber, B.L., Garber, J.E., Birch, J.M. et al. (1995) An evaluation of genetic heterogeneity in 145 breast-ovarian cancer families. Breast Cancer Linkage Consortium. *Am. J. Hum. Genet.*, 56, 254-264.
4. Fodor, F.H., Weston, A., Bleiweiss, I.J., McCurdy, L.D., Walsh, M.M., Tartter, P.L., Brower, S.T. and Eng, C.M. (1998) Frequency and carrier risk associated with common BRCA1 and BRCA2 mutations in Ashkenazi Jewish breast cancer patients. *Am. J. Hum. Genet.*, 63, 45-51.
5. Struwing, J.P., Hartge, P., Wacholder, S., Baker, S.M., Berlin, M., McAdams, M., Timmerman, M.M., Brody, L.C. and Tucker, M.A. (1997) The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews. *N. Engl. J. Med.*, 336, 1401-1408.
6. Hopper, J.L., Southey, M.C., Dite, G.S., Jolley, D.J., Giles, G.G., McCredie, M.R., Easton, D.F. and Venter, D.J. (1999) Population-based estimate of the average age-specific cumulative risk of breast cancer for a defined set of protein-truncating mutations in BRCA1 and BRCA2. Australian Breast Cancer Family Study. *Cancer Epidemiol. Biomarkers Prev.*, 8, 741-747.
7. Satagopan, J.M., Offit, K., Foulkes, W., Robson, M.E., Wacholder, S., Eng, C.M., Karp, S.E. and Begg, C.B. (2001) The lifetime risks of breast cancer in Ashkenazi Jewish carriers of BRCA1 and BRCA2 mutations. *Cancer Epidemiol. Biomarkers Prev.*, 10, 467-473.
8. Peto, J., Collins, N., Barfoot, R., Seal, S., Warren, W., Rahman, N., Easton, D.F., Evans, C., Deacon, J. and Stratton, M.R. (1999) Prevalence of BRCA1

- and BRCA2 gene mutations in patients with early-onset breast cancer. *J. Natl. Cancer Inst.*, 91, 943-949.
9. Claus, E.B., Schildkraut, J., Iversen, E.S., Jr., Berry, D. and Parmigiani, G. (1998) Effect of BRCA1 and BRCA2 on the association between breast cancer risk and family history. *J. Natl. Cancer Inst.*, 90, 1824-1829.
  10. Group, T.A.B.C.S. (2000) Prevalence of BRCA1 and BRCA2 mutations in a large population based series of breast cancer cases. *Br. J. Cancer*, 83, 1301-1308.
  11. Antoniou, A.C., Pharoah, P.D., McMullan, G., Day, N.E., Ponder, B.A. and Easton, D. (2001) Evidence for further breast cancer susceptibility genes in addition to BRCA1 and BRCA2 in a population-based study. *Genet. Epidemiol.*, 21, 1-18.
  12. Dunning, A.M., Healey, C.S., Pharoah, P.D., Teare, M.D., Ponder, B.A. and Easton, D.F. (1999) A systematic review of genetic polymorphisms and breast cancer risk. *Cancer Epidemiol. Biomarkers Prev.*, 8, 843-854.
  13. Shugart, Y.Y., Cour, C., Renard, H., Lenoir, G. and Goldgar, D. (1999) Linkage analysis of 56 multiplex families excludes the Cowden disease gene PTEN as a major contributor to familial breast cancer. *J. Med. Genet.*, 36, 720-721.
  14. Rahman, N., Teare, M.D., Seal, S., Renard, H., Mangion, J., Cour, C., Thompson, D., Shugart, Y., Eccles, D., Devilee, P. et al. (2000) Absence of evidence for a familial breast cancer susceptibility gene at chromosome 8p12-p22. *Oncogene*, 19, 4170-4173.
  15. Phelan, C.M., Rebbeck, T.R., Weber, B.L., Devilee, P., Rutledge, M.H., Lynch, H.T., Lenoir, G.M., Stratton, M.R., Easton, D.F., Ponder, B.A. et al. (1996) Ovarian cancer risk in BRCA1 carriers is modified by the HRAS1 variable number of tandem repeat (VNTR) locus. *Nat. Genet.*, 12, 309-311.
  16. Rebbeck, T.R., Wang, Y., Kantoff, P.W., Krithivas, K., Neuhausen, S.L., Godwin, A.K., Daly, M.B., Narod, S.A., Brunet, J.S., Vesprini, D. et al. (2001) Modification of brca1- and brca2-associated breast cancer risk by aib1 genotype and reproductive history. *Cancer Res.*, 61, 5420-5424.
  17. Rebbeck, T.R., Kantoff, P.W., Krithivas, K., Neuhausen, S., Blackwood, M.A., Godwin, A.K., Daly, M.B., Narod, S.A., Garber, J.E., Lynch, H.T. et al. (1999) Modification of BRCA1-associated breast cancer risk by the polymorphic androgen-receptor CAG repeat. *Am. J. Hum. Genet.*, 64, 1371-1377.
  18. Kadouri, L., Easton, D.F., Edwards, S., Hubert, A., Kote-Jarai, Z., Glaser, B., Durocher, F., Abeliovich, D., Peretz, T. and Eeles, R.A. (2001) CAG and GGC repeat polymorphisms in the androgen receptor gene and breast cancer susceptibility in BRCA1/2 carriers and non-carriers. *Br. J. Cancer*, 85, 36-40.
  19. Menin, C., Banna, G.L., De Salvo, G., Lazzarotto, V., De Nicolo, A., Agata, S., Montagna, M., Sordi, G., Nicoletto, O., Chieco-Bianchi, L. et al. (2001) Lack of association between androgen receptor CAG polymorphism and familial breast/ovarian cancer. *Cancer Lett.*, 168, 31-36.
  20. Tirkkonen, M., Johannsson, O., Agnarsson, B.A., Olsson, H., Ingvarsson, S., Karhu, R., Tanner, M., Isola, J., Barkardottir, R.B., Borg, A. et al. (1997) Distinct somatic genetic changes associated with tumor progression in carriers of BRCA1 and BRCA2 germ-line mutations. *Cancer Res.*, 57, 1222-1227.
  21. Tirkkonen, M., Tanner, M., Karhu, R., Kallioniemi, A., Isola, J. and Kallioniemi, O.P. (1998) Molecular cytogenetics of primary breast cancer by CGH. *Genes Chromosomes Cancer*, 21, 177-184.
  22. Sobel, E. and Lange, K. (1996) Descent graphs in pedigree analysis: applications to haplotyping, location scores, and marker-sharing statistics. *Am. J. Hum. Genet.*, 58, 1323-1337.
  23. Reimer, C.L., Borras, A.M., Kurdastani, S.K., Garreau, J.R., Chung, M., Aaronson, S.A. and Lee, S.W. (1999) Altered regulation of cyclin G in human breast cancer and its specific localization at replication foci in response to DNA damage in p53 +/+ cells. *J. Biol. Chem.*, 274, 11022-11029.
  24. Master, S.R., Hartman, J.L., D'Cruz, C.M., Moody, S.E., Ha, S.I., Belka, G.K., Chodosh, L.A. Functional microarray analysis of mammary development reveals a novel role in adaptive thermogenesis. *Mol. Endocrinol.*, (in press)
  25. Easton, D.F., Ponder, M.A., Huson, S.M. and Ponder, B.A. (1993) An analysis of variation in expression of neurofibromatosis (NF) type 1 (NF1): evidence for modifying genes. *Am. J. Hum. Genet.*, 53, 305-313.
  26. Webster, A.R., Richards, F.M., MacDonald, F.E., Moore, A.T. and Maher, E.R. (1998) An analysis of phenotypic variation in the familial cancer syndrome von Hippel-Lindau disease: evidence for modifier effects. *Am. J. Hum. Genet.*, 63, 1025-1035.
  27. Ford, D., Easton, D.F., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D.T., Weber, B., Lenoir, G., Chang-Claude, J. et al. (1998) Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. *Am. J. Hum. Genet.*, 62, 676-689.
  28. O'Connell, J.R. and Weeks, D.E. (1998) PedCheck: a program for identification of genotype incompatibilities in linkage analysis. *Am. J. Hum. Genet.*, 63, 259-266.
  29. Davis, S., Schroeder, M., Goldin, L.R. and Weeks, D.E. (1996) Nonparametric simulation-based statistics for detecting linkage in general pedigrees. *Am. J. Hum. Genet.*, 58, 867-880.
  30. Kruglyak, L., Daly, M.J., Reeve-Daly, M.P. and Lander, E.S. (1996) Parametric and nonparametric linkage analysis: a unified multipoint approach. *Am. J. Hum. Genet.*, 58, 1347-1363.